Constitutive p38HOG Mitogen-activated Protein Kinase Activation Induces Permanent Cell Cycle Arrest and Senescence

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ABSTRACT

Cellular senescence, initially observed during subculturing of normal diploid fibroblasts, can also be induced by chronic exposure to cellular stress, such as UV light, oxidative stress, or DNA damaging agents. Here we demonstrate that stable expression of an activated form of MKK6 (M KK6EE), a direct activator of the stress-induced p38HOG mitogen-activated protein kinase pathway, is sufficient for inducing features of senescence including a flattened, vacuolated, and irregular morphology, staining for acidic β-galactosidase, and accumulation of age-associated pigments. Consistent with the senescent phenotype, p38HOG activation induces a G1 cell cycle arrest, which is permanent and irreversible after 4 days. MKK6EE also induces biochemical features of senescence in a p53-dependent manner, including enhanced expression of p21 

Although it is clear that some extracellular signals can induce premature senescence, the molecular pathways involved in these responses are largely unknown. The MAPKs are key signal transduction pathways that couple extracellular signals to changes in gene expression. At least five different MAPK pathways have been identified, which respond to distinct environmental signals having various substrate specificities mediating separate physiological effects. The prototypical MAPK pathway, ERK1/2, is activated by growth factors, hematological cytokines, and neurotrophins. In contrast, two MAPK pathways, the SAPKs, also known as the JNKs, and the p38HOG kinases, are poorly activated by most growth factors but respond to multiple cytotoxic stresses, including UV irradiation, chemotherapy, heat shock, and inflammatory cytokines. As many of the stresses that activate SAPKs and p38HOG kinases can also induce premature senescence, we have examined the role of this process. To address their role in senescence specifically, we selectively activated p38HOG or SAPK in U2OS cells. We find that activation of p38HOG, but not SAPK, induces profound growth inhibition, and morphological, biochemical, and genetic features of senescence.

INTRODUCTION

The capacity of normal human cells to divide in culture is limited. For example, in normal human fibroblasts, proliferation decreases and finally halts after only 50 population doublings (1). This process of cellular ageing, called cellular senescence, contrasts to the high proliferative capacity of transformed, cancerous cells, which can be maintained in culture indefinitely (2). Senescence is characterized by physiological, structural, biochemical, and molecular changes. Aged cells in culture are typically larger, flatter, irregular, vacuolated, and accumulate dense lysosomal bodies containing fluorescent pigments (3, 4). Senescent cells fail to respond to growth factors or mitogens, and are permanently and irreversibly arrested at the G1 or G2 phases of the cell cycle (reviewed in Ref. 5). Biochemically, they show evidence of oxidative stress with damaged DNA and accumulation of modified proteins (6, 7). This phenotype is characterized by the increased expression of several genes, including those encoding extracellular matrix proteins and cell cycle genes such as the cdk inhibitors p16 and p21WAF1/Cip1/Sdi1, and cyclins D and E (5, 8).

Cellular senescence, initially observed during subculturing of normal diploid fibroblasts, can also be induced by hyperactivation of mitogenic pathways (9), chronic exposure to cellular stress, such as UV light, oxidative stress (10), or DNA damaging agents (11). This “premature” senescence is indistinguishable from cellular senescence induced by serial passaging of human cells, suggesting that cellular senescence represents a fate choice influenced by extracellular and intracellular signal transduction pathways.

MATERIALS AND METHODS

Reagents. The wild-type and kinase-dead (KA) human MKK6b cDNAs were kindly provided by Denis Templeton (Case Western Reserve University, Cleveland, OH). Dominant-positive MKK6EE was generated by substituting the acidic residue glutamic acid for each of Ser-207 and Thr-211, and adding antibiotic resistance genes and a CMV promoter. The plasmids were purified using the Qiagen Endo-Free kit, and sequences were verified. pCMV-cdk2 (pcMV-cdk2) was a kind gift of E. Harlow (Massachusetts General Hospital, Charleston, MA). cdk2dn IRESpuro2 was obtained by subcloning the BamHI insert of pCMVcdk2 into pIRESpuro2. Gal4 fusions of ATF2, c-jun, and Elk1, and plasmids coding MEKK1, MEKK1Δ, and the GAL4 promoter was from the PathDetect system (Stratagene). pSVβ-gal was obtained from Promega. All of the plasmids were purified using the Qiagen Endo-Free kit, and sequences were verified.

Transfections and Western Blotting. U2OS cells were transfected using Fugene-6 reagent, as described by the manufacturer (Roche). Where appropriate, cells were selected in puromycin (3 μg/mL) 2 days after transfection. In all of the transfections, a constant amount of DNA was used including the appropriate empty vector. Cell extracts were prepared using NP40 lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP40). Western blotting for cell cycle regulatory proteins was performed as described by the manufacturer.
150 mm NaCl, 1 mm EDTA, 1 mm DTT, 0.5% NP40, 5 mm NaF, 0.5 mm NaVO<sub>4</sub>, 1 µg/ml aprotinin, and 1 µg/ml leupeptin). After incubation on ice (30 min), lysates were cleared by centrifugation at 10,000 × g. Protein content of lysates was normalized by the Bradford method (Bio-Rad). For Western blotting, equal amounts of protein were separated by SDS-PAGE and blotted on Immobilon-P (Millipore), and incubated with antibodies as indicated. Antibodies to the following molecules were used: phospho-p38<sup>HOG</sup> (NEB), p38<sup>HOG</sup> (Santa Cruz), FLAG M2 (Sigma), α-tubulin (Sigma), p21 (Santa Cruz), cyclin E (HE12; BD PharMingen), cyclin D (DCS-6; BioMol), p53 (clone 1801; provided by S. Benchimol, Ontario Cancer Institute), cyclin A (Santa Cruz), and pRb (14001A, BD PharMingen).

### Colony Formation and Growth Curves

Colony formation assays have been used to demonstrate senescence-like effects induced by the retinoblastoma protein (13). We used a modified protocol to assess the effect of p38 on senescence. Cells (3 × 10<sup>3</sup>) were plated in 100-mm dishes, transected with the indicated plasmid, and selected as described above. For coactivation of p38 and SAPK, we transfected MKK6EE, MKK7EE, and SAPK in a 1:3:3 molar ratio. Two weeks later, cells were stained with 50% methanol and 1.5% crystal violet. Colonies were defined as >20 cells. Single viable cells were also counted as indicated. Parallel plates were transfected and selected for analysis of expression and activation of individual MAPks.

To measure cell growth, antibiotic selected cells were trypsinized and replated in 24-well dishes (1 × 10<sup>3</sup> cells/well). At the indicated times, cells were washed twice with PBS, fixed in 10% formaldehyde for 10 min, and rinsed with distilled water. Cells were stained with 0.1% crystal violet for 30 min, rinsed extensively, and allowed to dry. Dye was extracted from cells in each well with 10% acetic acid (200 µl) for 30 min. Absorbance at 595 nm was measured in 96-well microtiter plates in triplicate.

SA-βgal activity was determined using the standard protocol (3, 14). As a positive control, BJ fibroblasts were serially subcultured until senescent (population doubling ~85). BJ cells expressing the telomerase subunit (BJ/TERT) were a kind gift of Homayoun Varzi (Whitehead Institute, Boston, MA).

### Luciferase Assays

Luciferase and β-galactosidase assays were performed in triplicate using the Dual-Light system (Tropix, Foster City, CA) with protocols recommended by the manufacturer. Luciferase values were normalized for transfection efficiency by β-galactosidase readings. Results are expressed as averages of normalized values for at least three replicates.

### Flow Cytometry

Puromycin-selected cells were pulsed for 2 h with BrdUrd (10 µM Sigma) before trypsinization, washing, and resuspension in PBS. Cells were permeabilized and fixed using the BrdUrd fluorescence-activated cell sorter kit (Becton Dickinson). Flow cytometry was done using a Becton-Dickinson FACScan or FACScalibur, using 488-nm excitation, and the FL1 (BrdUrd) and FL3 (PI) filters. At least 10,000 cells were analyzed per sample.

To detect autofluorescence associated with lipofuscin accumulation, puromycin-selected cells were trypsinized, washed, and examined using 488-nm excitation and the FL1 filters as described (15).

### Stress-induced Senescence

U2OS cells were plated in six-well plate at a density of 1 × 10<sup>4</sup> cells/well. Wells containing 10 µM of SB202190 or vehicle control were treated with 150 µM H<sub>2</sub>O<sub>2</sub> or irradiated at 10 Gy. After 2 h, peroxide was removed, and cells were washed in PBS and recultured in fresh medium. SB202190 was replaced every day. After 7 days, cells were stained for SA-βgal. For the γ-ray experiment, both mock and irradiated were incubated for 11 days. Cells were then stained for SA-βgal activity as above.

Northern Blotting and Microarray Analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen) after 6 days of puromycin selection. Denatured RNA was resolved on 1.2% agarose-formaldehyde gels, transferred to Hybond nylon membranes (Amersham) and cross-linked using a Stratalinker (Stratagene). Probes were generated from p21 or 36B4 cDNA templates using the Random Priming kit (Amersham). Microarrays containing 19,200 duplicated cDNA probes were obtained from the Ontario Microarray Consortium. Averaging of duplicate spots and arrays was performed using the Array-Analysis software<sup>4</sup> with a cutoff signal-to-noise ratio and a minimum intensity threshold of 2.0 SDs above mean background for both channels.

### RESULTS

#### Specific Activation of p38 by MKK6EE in U2OS Cells

Phosphorylation of MAPks at invariant tyrosine and threonine residues by dual specificity kinases is necessary and sufficient for their activation. Two such kinases, MKK6 and MKK3, specifically phosphorylate the p38<sup>HOG</sup> MAPks but not ERK or SAPK (17). Overexpression of MKK6EE, a constitutively active version of MKK6, induces p38<sup>HOG</sup> phosphorylation in the absence of additional stimulation (17).

To confirm the specificity of MKK6EE we examined the activation of several MAPK-pathway responsive transactivating domains. The transactivation domains of ATF2, Elk-1, and c-Jun are physiologically phosphorylated by p38<sup>HOG</sup>, ERK, and SAPK, respectively. Phosphorylated transactivation domains, when expressed as fusions with the GAL4 DNA-binding domain, activate a coexpressed promoter containing five GAL4 DNA-binding sites upstream of the luciferase gene. As expected, expression of MKK6EE in U2OS cells increased the activity of GAL4-ATF2 ~4.5-fold, whereas kinase-dead MKK6 slightly reduced basal activity (Fig. 1). Addition of SB202190, a specific p38α/β inhibitor reduced the effect of MKK6EE to near basal levels (Fig. 1) but did not affect expression of MKK6EE (data not shown). MKK6EE had no significant effect on either the ERK-responsive GAL4-Elk1 or SAPK-responsive GAL4-cJun chimeric transcription factors, although expression of control MEKEE or MEKK1 constructs activated ERK- and SAPK-responsive elements, respectively (Fig. 1). These results confirm the specificity of MKK6EE for p38<sup>HOG</sup>-induced transcription in U2OS cells.

#### Loss of Proliferation in Tumor Cells Expressing Activated p38<sup>HOG</sup>

Oxidative stress is associated with aging of many cell types and mediates premature cellular senescence (18, 19). As p38<sup>HOG</sup> is activated by oxidative stress, we examined whether its activation was sufficient to induce a senescent phenotype. First, to test its effect on growth, we coexpressed MKK6EE or a kinase-dead mutant with the puromycin resistance gene using a single promoter system, which separates two open reading frames with an internal ribosome entry site (Fig. 2 A). Use of this construct causes virtually all of the antibiotic-resistant transfected cells to express the gene of interest (20).

We examined the effect of expressing MKK6EE on the growth of U2OS cells, a well-studied osteogenic sarcoma cell line that retains...
both wild-type pRb and p53 (21, 22). Cells were transfected with pIRES MKK6EE or pIRES MKK6KA, and antibiotic-resistant colonies were counted after 2 weeks. Relative to cells transfected with empty vector alone or with MKK6KA, <6% of the MKK6EE-transfected cells formed colonies (Fig. 2). Incubation of cells with the p38-specific inhibitor SB202190 blocked the inhibitory effect of MKK6EE on cell proliferation (Fig. 2B).

Whereas MKK6EE-transfected cells did not result in proliferating colonies, many viable, non-dividing, puromycin-resistant single cell clones were observed (Fig. 2C). These cells exhibited increased size and morphology, similar to that observed in senescent cells. MKK6EE-transfected cells remain adherent, flat, and enlarged for at least 3 weeks (data not shown). By contrast, expression of dn-cdk2 eliminated all of the viable cells illustrating that the cell cycle effects induced by MKK6EE are qualitatively different from those of interfering mutants of cdk2. This latter result may indicate that sustained dn-cdk2 expression induces apoptosis, as has been reported recently in U2OS cells (23).

To examine the role of SAPK in cell proliferation, we made use of a constitutively active version of MKK7, a SAPK activator. Stable expression of MKK7EE alone (not shown) or in combination with SAPKα, SAPKβ, or SAPKγ isoforms increased colony formation in U2OS cells with demonstrated induction of SAPK phosphorylation (Fig. 2, inset). Thus, constitutive activation of p38HOG and SAPK has different effects on cell proliferation. We also examined the effect of coactivation of p38 and SAPK. Even with 3-fold molar excess of SAPK components, sustained MKK6EE expression induced senescence in U2OS indicating that prolonged p38 activation is dominant over SAPK.

To examine the cell cycle effects of MKK6EE, kinase-dead MKK6, or vector control transfection, puromycin-selected cells were pulsed with BrdUrd, and analyzed for DNA content and BrdUrd incorporation. As shown in Fig. 3, MKK6EE expression leads to a near complete loss of BrdUrd-positive cells, consistent with a G1/G0 cell cycle arrest. The effect was almost completely inhibited by pretreatment of cells with SB202190 (10 μM). Thus, constitutive MKK6EE expression inhibits cell proliferation through a mechanism requiring p38HOG/αβ activity.

Cell Cycle Arrest Is Permanent and Irreversible. As senescence is associated with permanent cell cycle arrest, we examined the reversibility of growth inhibition caused by MKK6EE. Puromycin-selected cells, transfected with pRES MKK6EE, kinase dead MKK6, or vector, were plated, and cell growth was assayed over 9 days using a biomass assay. Compared with vector control, kinase-dead MKK6-expressing cells showed a slight but reproducible proliferative advantage (Fig. 4A). MKK6EE-expressing lines showed no increase in cell number over 9 days, suggesting a sustained effect on cell growth. This arrest was reversed by addition of SB202190 at the time of the transfection, but subsequent removal of the inhibitor at any time rapidly induced growth suppression (Fig. 4A, Washout).

We next evaluated the minimal duration of p38HOG activation needed to produce a permanent cell cycle arrest. SB202190 was added to MKK6EE-expressing U2OS cells 0, 4, 6, or 8 days after transfec-
p38 Activation Induces Features of Cellular Senescence. Features of MKK6EE-expressing cells resemble those that have surpassed their proliferative capacity and have become senescent. Like MKK6EE-expressing cells, senescent cells have a flat, dendritic morphology, cease proliferation with G1 DNA content at subconfluent densities despite the presence of serum (reviewed in Ref. (5), and possess traits of MKK6EE-expressing cells resembling those that have surpassed their proliferative capacity and have become senescent. We observed that 40% of MKK6EE-expressing cells were strongly positive for SA-β-gal activity (SA-β-gal), which is not observed in terminally differentiated or quiescent cells. We observed that 40% of MKK6EE-expressing cells have increased endogenous β-galactosidase activity (SA-β-gal), which is not observed in terminally differentiated or quiescent cells. Cellular aging is also associated with lysosomal accumulation of the autofluorescent pigment, lipofuscin (4). We examined the accumulation of lipofuscin-associated autofluorescence in vector, MKK6EE-, or MKK6KA-expressing cells. At 6 days after transfection there was a marked increase in fluorescence of MKK6EE-expressing cells compared with controls (Fig. 5B). This effect was not observed with MKK6KA cells or MKK6EE-expressing cells, which were treated with SB202190.

MKK6EE Induces Gene Expression Consistent with Senescence. Senescent cells have a characteristic pattern of gene expression characterized by changes in extracellular matrix genes, proinflammatory cytokines, and neutrophil genes (8). To examine the effects of MKK6EE-induced gene expression we prepared RNA from MKK6EE or vector-transfected U2OS cells. cDNA prepared from the

RNA was labeled with dCTP-conjugated Cy5 or Cy3 fluorescent dyes and hybridized to a microarray representing 19,200 known transcripts and expressed sequence tags. We identified 63 genes that were reproducibly activated or repressed by 1.75-fold or greater (see Supplemental Information; some genes were represented by more than one clone). Among major changes (Table 1), MKK6EE mRNA itself was induced 5.6-fold and p21 1.9-fold, consistent with our Western and Northern analysis (Fig. 4). Strikingly, many of the genes that we found to be up-regulated after MKK6EE expression have been identified in cells from Werner Syndrome subjects (30). Specifically, we observed increases in the light chains of ferritin (4.8-fold), αB-crystallin (13-fold), extracellular matrix proteins, and neutrophil recruitment genes, which have been identified as senescence-associated genes (30–32). Collectively, our analysis indicates that p38 induces senescence of U2OS tumor cells that is similar genetically and morphologically to replicative senescence.

p38 Is Not Necessary for Stress-induced Senescence. The induction of ferritin by MKK6EE suggested that oxidative stress may share pathways with p38-induced senescence. Iron potentiates the generation of reactive hydroxyl species and leads to oxidative damage of proteins and lipids (33). As hydrogen peroxide and related oxidative damage also leads to senescence, we examined the requirement of p38 in this process. Treatment of U2OS cells with hydrogen peroxide lead to a dose-dependent accumulation of senescent cells, as identified by SA-β-gal staining. Although peroxide activated p38, pretreatment with SB202190 did not block senescent morphology or SA-β-gal staining (Fig. 7). We also examined the role of p38 in γ-irradiation and transforming growth factor β-induced (data not shown) senescence. Senescence induced by these agents also did not require p38 activity in U2OS cells (see Fig. 7; data not shown). Together, these results indicate that p38 is sufficient but not necessary for stress-induced senescence, suggesting a role for additional, parallel stress-induced senescence pathways.

DISCUSSION

We demonstrate that expression of activated MKK6 induces morphological, biochemical, and genetic markers of cellular senescence, including permanent cell cycle arrest, up-regulation of p21Cip1, SA-β-gal staining, and lipofuscin-associated autofluorescence. Senescence
induced by expression of MKK6EE was blocked by the specific p38 inhibitor, SB202190, and was not observed on overexpression of a kinase-dead MKK6 gene, indicating that our results are not because of a nonspecific response to overexpression or because of nonspecific effects of the inhibitor. As SB202190 is unable to inhibit p38δ or p38γ, the senescence program induced by activated MKK6 is dependent on p38α and/or p38β. Both of these isoforms are expressed in U2OS cells (34).

Interestingly, p38-dependent cell cycle arrest was irreversible after 4 days of MKK6EE expression, indicating that p38 induces a time-dependent physiological response. The basis for this delayed commitment is unclear. However, the duration and strength of MAPK signals is a determinant in their physiological effects in other contexts. For example, transient ERK activation leads to cell proliferation, whereas robust, sustained activity leads to terminal differentiation or senescence (9, 35–37). Similarly, transient SAPK activity is associated with proliferation, whereas extended activity may play a role in apoptosis (38).

The molecular effectors of p38-dependent senescence remain to be understood. Senescence induced by ERK pathway components in murine cells requires the cdk inhibitor p21Cip1 and p19ARF (9, 36, 37), and may also be dependent on p53 in some cases. In our experiments, MKK6EE induced p21 mRNA and protein, but U2OS cells do not express p16 or p19. Furthermore, p53 levels were not increased, and it appears not to be required for p38-dependent cell cycle arrest (data not shown). The retinoblastoma protein may be critical, as it is involved in senescence-associated expression of fi-

Fig. 5. MKK6EE induces morphological and histochemical features of premature senescence. A, U2OS cells were stained with SA-β-gal after 5 days selection in puromycin. The proportion of MKK6EE-expressing cells that stained positive was between 40 and 60%. As controls for staining normal, senescent human BJ fibroblasts or BJ fibroblasts, telomerase (TERT) were stained. B, autofluorescence of cells was examined 6 days after transfection. The percentage of cells within gate (M1) and the mean fluorescence intensities are noted for each sample.

Table 1 Changes in senescent markers in cells constitutively expressing MKK6EE

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of spots passed</th>
<th>Average fold activation</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>17</td>
<td>7.2</td>
<td>0.9</td>
</tr>
<tr>
<td>MKK6</td>
<td>6</td>
<td>5.7</td>
<td>0.4</td>
</tr>
<tr>
<td>αB-crystallin</td>
<td>21</td>
<td>5.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Ferritin, light chain</td>
<td>6</td>
<td>4.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Protein phosphatase 5, catalytic subunit</td>
<td>6</td>
<td>4.6</td>
<td>0.6</td>
</tr>
<tr>
<td>MAPK-activated protein kinase 2</td>
<td>6</td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Interleukin 8</td>
<td>4</td>
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<td>1.1</td>
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<td>Osteonectin</td>
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<td>1.1</td>
</tr>
<tr>
<td>Collagenase</td>
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<td>0.6</td>
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<tr>
<td>α1(I) collagen</td>
<td>12</td>
<td>1.8</td>
<td>0.6</td>
</tr>
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</table>

Fig. 6. Expression of cell cycle-regulated genes in stably transfected cells. Protein (A) or RNA (B) was extracted from U2OS cells expressing empty vector or MKK6. SB202190 was added at time of transfection and maintained the duration of the experiment. Samples were subjected to Western or Northern blotting.
branectin and osteonectin, genes that are also up-regulated by MKK6EE (39).

The relationship between p38- and ERK-induced senescence also remains to be explored. Morooka and Nishida (40) have demonstrated that expression of MEK results in p38 activation and neurite outgrowth, which is blocked by SB23580. These surprising results indicate an essential role for p38 in ERK-mediated physiological effects. However, we have not been able to observe any activation of p38 by activated MEK in U2OS cells, and expression of MKK6EE appears to inhibit, not increase, the activity of ERK (data not shown).

Telomerase may also be a central regulator of senescence because expression of its catalytic subunit is sufficient to extend the life of activated MEK in U2OS cells, and expression of MKK6EE appears to cancel an essential role for p38 in ERK-mediated physiological effects.

To relate the effects of MKK6EE expression to senescence at the molecular level, we examined global gene expression by microarray analysis. The results of this analysis demonstrated considerable similarity of gene expression of MKK6EE-expressing cells and senescent fibroblasts. The most highly induced genes include fibronectin, an essential extracellular matrix component that is overexpressed in senescent pig or human fibroblasts (30, 44–46). Collagen and osteonectin, which are up-regulated by MKK6EE, are also induced in cells from patients with Werner Syndrome and in late passage fibroblasts (30, 44, 47). Collagenase is also differentially expressed in MKK6EE-expressing cells and in other models of senescence suggesting that extracellular matrix can change growth potential and may contribute to the appearance of these cells (48, 49). Osteonectin, which is also up-regulated by MKK6EE, can inhibit cell cycle progression, although its relation to p38HOG-induced G1 arrest remains to be defined (50).

Previous reports have implicated cellular stress in senescence in both normal (39) and immortalized cell types (11, 51). Exposure of fibroblasts to hydrogen peroxide induces irreversible growth arrest and morphological changes resembling senescence (10). Wang et al. (51) reported that treatment of nasopharyngeal-transformed cells with cis-platinum induces senescence. As p38 is activated by these and other stresses, it may be a mediator of such stress-induced senescence. p38 may also play a role in senescence induced by repeated passaging, as senescent skeletal muscle demonstrates hallmarks of a stress response, including expression of known p38 targets, such as MAPKAPK-2 (31).

Our results suggest that cellular senescence represents a fate decision that can be modified by intracellular signal transduction pathways. As we were able to induce senescence in tumor cells, genetic changes that have occurred in U2OS cells are apparently not sufficient to prevent p38HOG-induced senescence. We suggest that sustained activation of p38 in cancer cells may provide a novel therapeutic approach. Conversely, use of specific p38HOG inhibitors may delay replicative senescence and aging.

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